pCDH cDNA Cloning and Expression Lentivectors

Cat. #s CD500 – CD700

User Manual

Store kit at -20°C on receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 5-082212)

Contents

I. Introduction and Background
A. Purpose of this Manual 2
B. Advantages of the Lentivector Expression System 2
C. pCDH cDNA Cloning and Expression Lentivectors 3
D. List of Components 7
E. Additional Required Materials 7
F. Safety Guidelines 9

II. Protocol
A. cDNA Amplification 11
B. Primer Design for T2A Vector Cloning 11
C. Preparation of Digested pCDH Vectors 12
D. Cloning of cDNA into pCDH Vectors 13
E. Packaging of pCDH Expression Construct 15

III. Troubleshooting
A. Large number of colonies on control plate 16
B. No or low number of colonies on plate with cDNA sample 16
C. No correct cDNA inserts 17

IV. References 18

V. Appendix
A. Maps and Features for pCDH Vectors 19
B. Descriptions of Features in pCDH Vectors 22
C. Properties of copGFP Fluorescent Protein 23
D. Related Products 23
E. Technical Support 24

VI. Licensing and Warranty Statement 25

I. Introduction and Background

A. Purpose of this Manual

This manual provides details and information necessary to generate expression constructs of your gene of interest in the pCDH cDNA Cloning and Expression Lentivectors. Specifically, it provides critical instructions on amplification and cloning cDNA into the pCDH vectors, and verification of the final expression constructs. This manual does not include information on packaging the pCDH expression constructs into pseudotyped viral particles or transducing your target cells of choice with these particles. This information is available in the user manual Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells which is available on the SBI website (www.systembio.com). Before using the reagents and material supplied with this system, please read the entire manual.

B. Advantages of the Lentivector Expression System

Lentiviral expression vectors are the most effective vehicles for the delivery and expression of a gene of interest to almost any mammalian cell—including non-dividing cells and model organisms (C.A. Machida, 2003; M. Federico, 2003; W. C. Heiser, 2004). As with standard plasmid vectors, it is possible to introduce lentivector expression constructs in plasmid form into the cells with low-to-medium efficiency using conventional transfection protocols. However, by packaging the lentivector construct into viral particles, you can obtain highly efficient transduction of expression constructs—even with the most difficult to transfect cells, such as primary, stem, and differentiated cells. The expression construct transduced in target cells is integrated into genomic DNA and provides stable, long-term expression of the target gene.

SBI offers a third generation of the most popular HIV-1 based lentivector expression system which consists of three main components:

(1) The lentiviral expression vector (*e.g.*, pCDH-EF1-MCS-T2A-Puro)

(2) The lentiviral packaging plasmids (*e.g.*, pPACKH1™ Packaging Plasmid mix)

(3) A pseudoviral particle producer cell line (*e.g.*, 293TN cells)

The expression lentivector contains the genetic elements responsible for packaging, transduction, stable integration of the viral expression construct into genomic DNA, and expression of the target gene sequence. The packaging vector provides all the proteins essential for transcription and packaging of an RNA copy of the expression construct into recombinant viral particles. To produce a high titer of viral particles, expression and packaging vectors are transiently co-transfected into producer mammalian cells (*e.g.*, HEK 293 cells). For a detailed description of SBI's Lentivector expression system, please refer to the Lentivector Expression System user manual.

C. pCDH Cloning and Expression Lentivectors

SBI provides a collection of cDNA cloning and expression vectors for various applications (Table 1). A gene of interest can be cloned under a CMV or EF1 promoter with or without another expression cassette for a reporter gene (copGFP or Puro^R). Genes can be either expressed transiently through transfection or stably expressed in a target cell line through transduction with packaged viral particles.

	cDNA vectors	target gene promoter	Expression level	Application
Single Promoter	pCDH-EF1-MCS	EF1	Medium	robust in most cell types, including primary differentiated cells
Single Promoter	pCDH-EF1-MCS-T2A-Puro	EF1		
Single Promoter	pCDH-EF1-MCS-T2A-copGFP	EF1		
Single Promoter	pCDH-MCS-T2A-Puro-MSCV	MSCV	High	hematopoietic / stem cell lines
Single Promoter	pCDH-MCS-T2A-copGFP-MSCV	MSCV		
Single Promoter	pCDH-CMV-MCS	CMV	High	commonly used cell lines (*e.g.* HeLa, HEK293, HT1080, H1299)
Single Promoter	pCDH-CMV-MCSr	CMV		
Dual Promoter	pCDH-CMV-MCS-EF1-Puro	CMV		
Dual Promoter	pCDH-CMV-MCS-EF1-copGFP	CMV		

Table 1. pCDH Vector Applications. Comparison of the expression levels of different promoters and the various applications proposed for each cDNA vector.
EF1: elongation factor 1α; MCS: multiple cloning sites; T2A: self-cleavable 2A peptide; MSCV: 5'LTR promoter from mouse stem cell virus; CMV: cytomegalovirus promoter.

Choice of Promoter

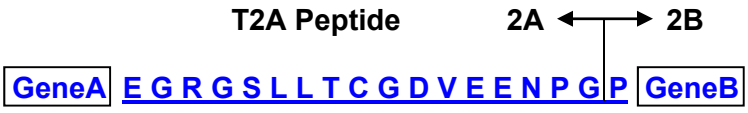
The major concern of cDNA expression in lentivectors is the efficiency level and stability of expression in target cell lines. The Cytomegalovirus (CMV) promoter is a strong and most commonly used viral promoter that constitutively expresses downstream genes. While the CMV promoter works perfectly in the most common cell lines, it shows poor expression in some stem cell lines and hematopoietic cell lines (R.F. Doll, 1996; E.D. Papadakis, 2004). The housekeeping elongation factor 1α (EF1) promoter has been shown to exceed and outlast CMV-mediated expression in retroviral, lentiviral, and adenoviral vectors, in hematopoietic cell lines (K. Tokushige 1997; H. Nakai, 1998; C. Teschendorf, 2002). EF1 also performs well in most common cell lines.

MSCV promoter is the 5'-LTR promoter of murine stem cell virus. When a portion of the U3 region of the 3' HIV LTR was replaced with the U3 region of MSCV LTR, the resulted hybrid HIV/MSCV LTR has dramatically increased the transgene expression level in human CD34+ hematopoietic cells (J.K. Choi, 2001). After integration into genomic DNA, this promoter transcribes a long transcript with an intron in the 5'UTR flanked with splice donor and acceptor sites derived from the lentiviral vector. Further studies found that additional CpG mutations in the MSCV LTR reduced transcriptional silencing in embryonic stem cells (C.S. Swindle, 2004). We constructed cDNA expression vectors with the CpG-deficient MSCV incorporated into the 3' HIV LTR. After integration into genomic DNA, 3'MSCV/LTR will replace the 5'LTR and provide a high level of expression of the target gene and reporter gene downstream.

2A Peptide-enabled dual expression system

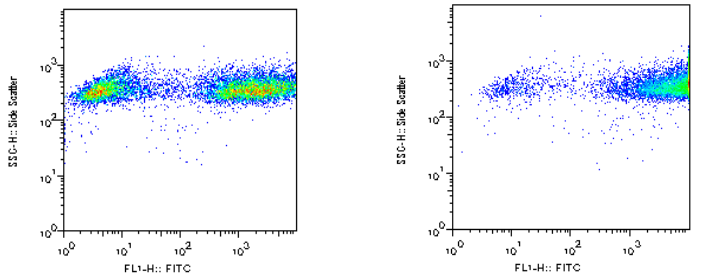
Coexpression of a reporter gene together with a gene of interest is a useful approach for selecting transfected or transduced cells. This is commonly achieved by using two independent internal promoters, such as CMV and EF1 in pCDH-CMV-MCS-EF1-copGFP, or by linking two transgenes with an internal ribosomal entry site (IRES) element in a single bicistronic transcript. Many dual promoter pairs have shown a high level of expression of both transgenes in standard cell lines—however, promoter interference often occurs in some cell lines. There are also two main problems that limit the use of IRES: the large size and the imbalanced expression between the first and second cistrons (H. Mizuguchi, 2000; X.Yu, 2003).

The "self-cleaving" 2A peptides have been used successfully to generate multiple proteins from a single promoter in many applications (P. de Felipe, 2004; M.J. Osborn, 2005; P. de Felipe, 2006). The 2A-like sequences exist in several viruses and are used to mediate protein cleavage from a single open reading frame. Through a ribosomal skip mechanism, the 2A peptide prevents normal peptide bond formation between the 2A glycine and the 2B proline without affecting the translation of 2B (M.L. Donnelly, 2001):



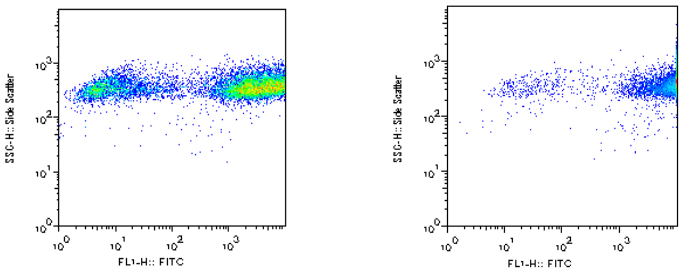
SBI's cDNA expression vectors incorporate the 2A-like sequence (T2A) from the insect virus *Thosea asigna* to mediate the coexpression of a reporter gene with the target cDNA. Reporter genes have been cloned at either the first or second positions, and we achieved high expression levels at both locations (see Figure 1).

A. **B.**



pCDH-EF1-**puro**-T2A-copGFP pCDH-**puro**-T2A-copGFP-MSCV

C. **D.**



pCDH-EF1-**cG**-T2A-Puro pCDH-**cG**-T2A-Puro-MSCV

Fig. 1. Flow cytometry analysis of HT1080 cells transduced with dual reporter constructs. The puromycin-resistance gene (puro) was cloned into pCDH-EF1-MCS-T2A-copGFP **(A)** and pCDH-MCS-T2A-copGFP-MSCV **(B)**; and the copGFP gene (cG) was cloned into pCDH-EF1-MCS-T2A-Puro **(C)** and pCDH-MCS-T2A-Puro-MSCV **(D)**. The resulting dual reporter constructs were packaged into pseudoviral particles followed by transduction into HT1080 cells. All constructs were also puromycin resistant (data not shown).

The HIV-1 derived pCDH vectors contain the following common features:

- **Multiple Cloning Site (MCS)—**for cloning the gene of interest in the MCS located downstream of the CMV promoter.
- **WPRE element—**enhances stability and translation of the CMV-driven transcripts.
- **SV40 polyadenylation signal—**enables efficient termination of transcription and processing of recombinant transcripts.
- **Hybrid RSV/5LTR promoter—**provides a high level of expression of the full-length viral transcript in producer 293 cells.
- **Genetic elements (cPPT, gag, env, LTRs)**—necessary for packaging, transducing, and stably integrating the viral expression construct into genomic DNA.
- **SV40 origin—**for stable propagation of the pCDH plasmid in mammalian cells.
- **pUC origin—**for high copy replication and maintenance of the plasmid in *E.coli* cells.
- **Ampicillin resistance gene—**for selection in *E.coli* cells.

D. List of Components

Component	Conc.	Amount
pCDH cDNA Expression Vector	0.5 μg/μl	20 μg

All plasmids are shipped at a concentration of 0.5 μg/μl and an amount of 20 μg. All plasmids are shipped in dry ice or blue ice and should be stored at -20°C upon receipt. Properly stored plasmids are stable for 12 months from the date received.

Available pCDH cDNA Cloning and Expression Lentivectors:

Vectors without reporter	Catalog #
pCDH-CMV-MCS *	CD500B-1
pCDH-CMV-MCSr **	CD501A-1
pCDH-EF1-MCS	CD502A-1

Vectors with reporter genes	Catalog #
pCDH-CMV-MCS-EF1-Puro †	CD510B-1
pCDH-CMV-MCS-EF1-copGFP ‡	CD511B-1
pCDH-EF1-MCS-T2A-Puro	CD520A-1
pCDH-EF1-MCS-T2A-copGFP	CD521A-1
pCDH-MCS-T2A-Puro-MSCV	CD522A-1
pCDH-MCS-T2A-copGFP-MSCV	CD523A-1

* new version of pCDH1-MCS1 (Cat. # CD500A-1)
** originally named pCDH1-MCS2 (Cat. # CD501A-1)
† new version of pCDH1-MCS1-EF1-Puro (Cat. # CD510A-1)
‡ new version of pCDH1-MCS1-EF1-copGFP (Cat. # CD511A-1)

For MCS sequences for the various vectors, please refer to Appendix.

E. Additional Required Materials

For Cloning

- Restriction enzymes for digestion of the vectors and/or inserts
  (Recommended: New England BioLabs enzymes)
- High Fidelity Long-distance PCR enzymes
- T4 DNA Ligase and ligation reaction buffer
  (Recommended: New England BioLabs T4 DNA Ligase (400 U/μl), Cat. # M0202S. Dilute to 40 U/μl in 1X ligation buffer with the provided 10X buffer just before use)
- High efficiency competent *E. coli* cells (RecA$^-$)
  (Recommended: MaxEfficiency Stbl2 competent cells, Life Tech (Cat # 10268-019) or One Shot OmniMAX 2 T1R competent cells, Cat. # C854003)
- Petri plates containing LB Agar media with 50 μg/ml Ampicillin

For Screening Inserts and Sequencing

- Taq DNA polymerase, reaction buffer, and dNTP mix
  (Recommended: Clontech Titanium™ Taq DNA polymerase, Cat. # 639208)
- PCR machine
- 2-3% 1X TAE Agarose gel

For Purifying cDNA Constructs after Cloning

- Plasmid purification kit
  (Recommended: QIAGEN Endofree Plasmid Maxi Kit, Cat. # 12362. The following kit combination can be used for Midi scale (up to 200 μg of plasmid DNA) preparation of endotoxin-free DNA:
  - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Buffer Set, Cat. # 19048

  Please visit the QIAGEN website to download the specialized protocol that is not contained in the current user manual:
  - http://www1.qiagen.com/literature/protocols/pdf/QP15.pdf

For Transfection of pCDH Constructs into Target Cells

- Transfection Reagent
  (Recommended: Invitrogen Lipofectamine 2000, Cat. # 11668-027)

For Packaging of pCDH Constructs in Pseudoviral Particles

- In order to package your pCDH cDNA constructs into VSV-G pseudotyped viral particles, you will need to purchase the pPACKH1 Lentivector Packaging Kit (Cat. # LV500A-1). The protocol for packaging and transduction of packaged pseudoviral particles is provided in the Lentivector Expression Systems User Manual.
- 293 Producer Cell Line
  (Recommended: SBI 293TN Cell Line, Cat. # LV900A-1 or ATCC 293 Cells, Cat. # CRL-11268)
- Transfection Reagent
  (Recommended: Invitrogen Lipofectamine, Cat. # 18324-111 and Plus Reagent, Cat # 11514-015).

F. Safety Guidelines

SBI's expression lentivectors together with the pPACK packaging plasmids comprise the third-generation lentiviral expression system. The HIV-based lentivectors are based on the vectors developed for gene therapy applications by Dr. J. G. Sodroski (U.S. patents # 5,665,577 and # 5,981,276).

Both FIV-based and HIV-based lentivector systems are designed to maximize their biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter (in HIV-based vectors) and CMV promoter (in FIV-based vectors) upstream of 5'LTR in the lentivector allow efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 that are used in this system.
- Number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (*gag, pol, rev*), and the corresponding proteins are expressed from different plasmids (for HIV-based packaging plasmids) lacking packaging signals and share no significant homology to any of the expression lentivectors, pVSV-G expression vector, or any other vector, to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (*gag, pol, rev)* will be present in the packaged viral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Pseudoviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of SBI's lentivectors falls within NIH Biosafety Level 2 criteria due to the potential biohazard risk of possible recombination with endogenous viral sequences to form self-replicating virus, or the possibility of insertional mutagenesis. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm. It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and always follow standard microbiological practices, which include:

- Wear gloves and lab coat all the time when conducting the procedure.
- Always work with pseudoviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.
- Please keep in mind that pCDH vectors are integrated into genomic DNA and could have a risk of insertional mutagenesis.

II. Protocol

The following section provides general guidelines for the cloning of cDNA, amplified by PCR, into pCDH vectors.

A. cDNA Amplification

Full length cDNA fragments can be recloned from another plasmid or amplified by PCR. PCR-based cloning is the most convenient way for full-length cDNA cloning in pCDH vectors. The cDNA lentivector does not contain an ATG initiation codon. A translation initiation sequence must be incorporated in the insert cDNA if the cDNA fragment to be cloned does not already have an ATG codon. We also recommend including a Kozak sequence (*i.e.* GCCACC) before the ATG for optimal translation. For amplification of the target cDNA fragment, design a 5'-primer (containing a Kozak sequence and ATG codon) and 3'-primer with unique restriction sites present in the MCS of the pCDH vector but not present in the cDNA sequence. Amplify the cDNA fragment by high fidelity long-distance PCR using about 200 ng of plasmid template DNA and a minimum number of cycles (usually 12-15 cycles), purify, digest the amplified product with end-specific restriction enzyme(s) and purify the digested PCR product in a 1.2% agarose gel to prevent contamination with the original plasmid used for amplification.

B. Primer Design for Cloning into Vectors with T2A Sequence

Since the gene of interest and the reporter gene in cDNA expression vectors containing a T2A peptide sequence will form one open-reading frame, extra attention should be paid when designing the 3' primer for amplifying the target sequence. First of all, do not include a stop codon at the 3' end of target sequence—this would prevent the expression of the reporter gene; secondly, place the target sequence in-frame with the 2A peptide. For example, if you would like to clone your target sequence between Xba1 and BstB1, you would need to add one more nucleotide at the end of your target sequence in order to make it in-frame with the 2A peptide and reporter gene (Figure 2).

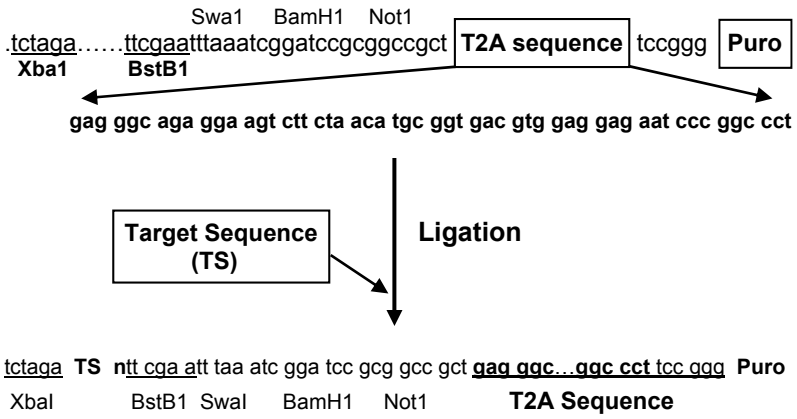

Swa1 BamH1 Not1
.tctaga……ttcgaatttaaatcggatccgcggccgct
Xba1 BstB1
T2A sequence
tccggg
Puro
gag ggc aga gga agt ctt cta aca tgc ggt gac gtg gag gag aat ccc ggc cct
Target Sequence (TS)
Ligation
tctaga TS ntt cga att taa atc gga tcc gcg gcc gct gag ggc…ggc cct tcc ggg Puro
XbaI BstB1 SwaI BamH1 Not1 T2A Sequence

Fig. 2. Sequence arrangement after target sequence is inserted between Xba1 and BstB1 in cDNA cloning vector pCDH-EF1-MCS-T2A-Puro. An additional nucleotide (n) is added after the last codon of the target sequence in order to keep it in frame with the T2A sequence.

C. Preparation of Digested pCDH Vector

Digest the pCDH vector with the corresponding restriction enzymes used in the preparation of the cDNA fragments, and then verify complete digestion of the vector by agarose gel electrophoresis. We suggest that you perform only preparative gel purification of the digested vector if more than one restriction enzyme is used. If you use a single restriction enzyme, dephosphorylation as well as gel purification of the vector is necessary to reduce the background in the vector ligation step.

D. Cloning of cDNA into pCDH Vector

The optimal insert-to-vector molar ratio may be different for different inserts. Always try at least two different ratios (*e.g.*, 10:1 and 30:1) for each experiment. Also make sure to include one negative control reaction, which contains only the digested vector.

1. Ligation of cDNA to Vector

a. Dilute the gel-purified, digested vector to 10 ng/μl.

b. Set up 10 μl ligation reactions for each sample and control as follows:

1.0 μl	Digested pCDH Vector (10 ng/μl)
7.0 μl	cDNA insert (usually 30-50 ng) or Nuclease-free water
1.0 μl	10X T4 DNA Ligase Buffer
1.0 μl	T4 DNA ligase (40 U/μl)
10.0 μl	Total volume

c. Incubate the ligation reactions at 16°C for 1-2 hrs if it is sticky-end ligation. For blunt-end ligation, use an overnight incubation.

2. Transform *E. coli* with the ligation product

Transform competent cells** (with a transformation efficiency of at least $1x10^9$ colonies/μg pUC19) with the whole ligation reaction (10 μl) following the protocol provided with the competent cells. Plate the transformed bacteria on LB-Ampicillin agar plates.

**Note: We recommend using Stbl2 or OmniMax 2 T1R competent cells for transformation and propagation of the lentivector construct to avoid unwanted lentivector recombination events.

3. Identify Clones with the cDNA Insert

a. Depending on the ratio of colony numbers for the cDNA sample vs. the negative control sample, randomly pick 5 or more well-isolated colonies and grow each clone in 100 μl of LB Broth with 75 μg/ml ampicillin at 30°C for 2 hours with shaking.

b. Use 1 μl of each bacterial culture for screening cDNA inserts by PCR and continue to grow the culture for another 4 hours. Store the culture at 4°C.

c. Prepare a PCR Master Mix with PCR primers flanking the cDNA insert:

1 rxn	10 rxn	Composition
0.5 µl	5 µl	PCR primer 1 (10 µM)
0.5 µl	5 µl	PCR primer 2 (10 µM)
0.5 µl	5 µl	50X dNTP mix (10 mM of each)
2.5 µl	25 µl	10X PCR Reaction Buffer
19.5 µl	195 µl	Nuclease-free water
0.5 µl	5 µl	Taq DNA polymerase (approx. 5 U/µl)
24.0 µl	240 µl	Total volume

d. Mix the master mix very well and aliquot 24 µl into each well of 96-well PCR plate or individual tubes.

e. Add 1 µl of each bacterial culture from step (b) into each well (or tube).

f. Proceed with PCR using the following program:

94°C, 4 min	1 cycle
94°C, 0.5 min, then 68°C, 1 min/1 kb*	25 cycles
68°C, 3 min	1 cycle

* depending on the size of final PCR product, use a shorter or longer time.

g. Take 5 µl of the PCR reaction and run it on a 1.2% agarose/EtBr gel in 1X TAE buffer to identify clones with correct insert.

Grow a positive clone with the cDNA insert in an appropriate amount of LB-Amp Broth, and purify the construct using an endotoxin-free plasmid purification kit (see Section I.E).

Confirm identity of the cDNA insert by sequence analysis of the construct using the one of the PCR primers. Alternatively, you may use one of the following sequencing primers which are located upstream of the MCS:

Vectors with CMV: 5'-CACGCTGTTTTGACCTCCATAGA-3'
Vectors with EF1: 5'-CTCCACGCTTTGCCTGACCCTGCTT-3'
Vectors with MSCV: 5'-GGGGTACAGTGCAGGGGAAAGAAT-3'

E. Packaging of the pCDH Expression Constructs into Pseudoviral Particles

If you are planning to create a stably transduced cell line expressing your gene of interest, you first need to package the cDNA lentiviral construct into lenti pseudoviral particles. For this purpose, you will need to purchase the pPACKH1 Lentivector Packaging Kit from SBI (see Appendix). Figure 3 schematically shows all steps which need to be performed in order to generate pseudoviral packaged cDNA expression constructs.

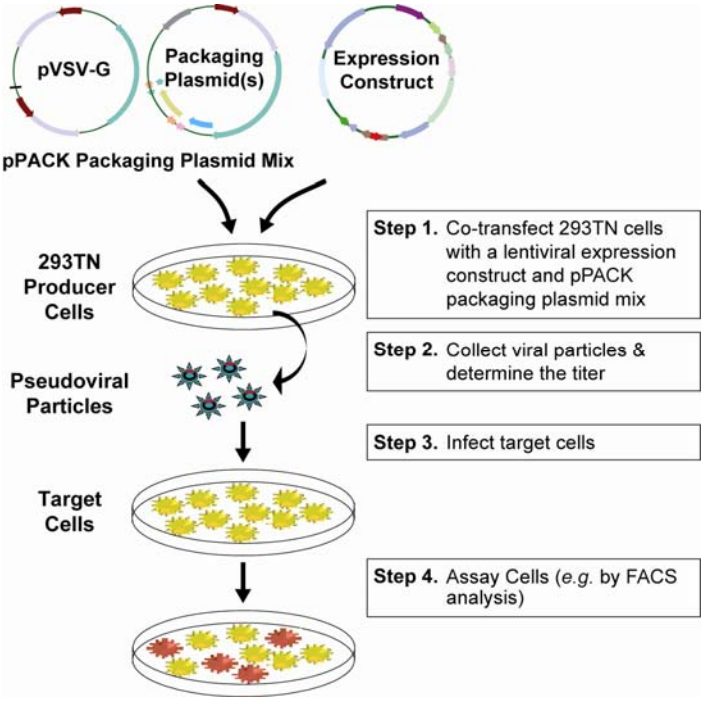

pVSV-G
Packaging Plasmid(s)
Expression Construct
pPACK Packaging Plasmid Mix
293TN Producer Cells
Step 1. Co-transfect 293TN cells with a lentiviral expression construct and pPACK packaging plasmid mix
Step 2. Collect viral particles & determine the titer
Pseudoviral Particles
Step 3. Infect target cells
Target Cells
Step 4. Assay Cells (e.g. by FACS analysis)

Fig. 2. Schematic presentation of the packaging procedure for lentivector expression constructs and making of stable cell lines.

The Lentivector Expression System User Manual includes the procedural information for packaging and transducing the expression constructs. This user manual is also available on the SBI web site (www.systembio.com). Although you can create stable transfectants with the lentiviral construct using standard transfection and selection protocols, transduction of the lentiviral cDNA construct using packaged pseudoviral particles is the most efficient way to deliver cDNA constructs in a wide range of cells, including dividing, non-dividing, and hard-to-transfect cells.

III. Troubleshooting

A. Large number of colonies on negative control plate

If you see that the colony number on the negative control plates (with no insert) is equal or more than on the plate with the cDNA sample, there is probably undigested plasmid contamination. Check your digestion conditions, and repeat digestion with an increased concentration of restriction enzyme(s) or use a longer reaction time. For best results, gel-purify and dephosphorylate the vector after single enzyme digestion. Also, check the sequences of the PCR primers in order to be sure that the necessary restriction sites are present.

B. No or low number of colonies on plate with cDNA sample

The efficiency of cDNA cloning into the pCDH vector depends on many factors, including size, purity, integrity, modification of insert, selection of restriction sites, etc. If your cDNA sample ligation resulted in only a few colonies, please continue with PCR screening first. If none of these few colonies has the right insert, or you did not get any colonies at all, it may be caused by:

1. Inappropriate ratio of insert-to-vector

Not enough or too much insert could inhibit the ligation reaction. Try a different ratio of insert-to-vector to optimize the ligation reaction. Sometimes, the yield of the ligation reaction may also be improved by increasing both the insert and vector amounts.

2. Low ligation efficiency

a.	Inactive ligase and /or ligase reaction buffer	Test your ligase and reaction buffer for activity using different vector and insert. Replace the reagents if they are proven inactive.
b.	Ligation inhibitors are present	EDTA and high salt may inhibit the ligation reaction.

3. Low transformation efficiency

a.	Low quality or poor handling of competent tested by transforming	Handle the competent cells gently. Many cells do not allow re-freezing cells after thawed. Quality of competent cells may be a circular plasmid to determine cell competency. Use competent cells with a transformation efficiency of at least $1x10^9$ colonies/μg of pUC19 plasmid.
b.	Wrong antibiotic or too much antibiotic in the media	The plates used for cloning should contain 50-100 μg/ml ampicillin in the media.

C. No correct cDNA inserts

If the colony number for the cDNA sample is more than for the negative control sample (*i.e.* vector only), but you failed to amplify cDNA insert, it could be that:

1. Inactive Taq polymerase

Test the activity of the PCR master mix by amplifying cDNA from the original template. Replace the PCR reagents if they are proven inactive.

2. Wrong primer was used

Make sure you are using the correct primers for the specific orientation of cDNA insert.

3. Not enough clones were screened

Pick more colonies for screening.

IV. References

C.A. Machida (Edit). Viral vectors for gene therapy. Methods and Protocols. (2003), Humana Press.

C.S. Swindle, H.G.Kim, and C.A.Klug. Mutation of CpGs in the murine stem cell virus retroviral vector long terminal repeat represses silencing in embryonic stem cells. J.Biol.Chem., (2004) 279: 34-41

E.D. Papadakis, S.A. Nicklin, A.H. Baker and S.J. White. Promoters and control elements: designing expression cassettes for gene therapy. Current Gene Therapy, (2004) 4: 89-113

J.K.Choi, N. Hoang, A.M. Vilardi, P.Conrad, S.G.Emerson, and A.M. Gewirtz. Hybrid HIV/MSCV LTR Enhances transgene expression of lentiviral vectors in human CD34+ hematopoietic cells. Stem Cells, (2001) 19:236-246

K.Tokushige, D. Moradpour, T. Wakita, M. Geissler, N. Hayash, and J.R. Wands. Comparison between cytomegalovirus promoter and elongation factor-1 alpha promoter-driven constructs in the establishment of cells expression hepatitis C virus core protein. J. Virol Methods. (1997) 64:73-80.

M. Federico (Edit). Methods in Molecular Biology. Volume 229. Lentivirus gene engineering protocols. (2003), Humana Press.

M.J. Osborn, A.P. Mortari, R.T. McElmurry, S.K. Bell *et al*. A picornaviral 2A-like sequence-based tricistronic vector allowing for high-level therapeutic gene expression coupled to a dual-reporter system. Molecular Therapy. (2005) 12:569-574

M.L. Donnelly *et al*. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage" mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal "skip". J. Gen. Virol. (2001) 82:1013-1025

P. de Felip, Skipping the co-expression problem: the new 2A "CHYSEL" technology. Genetic Vaccines and Therapy. (2004) 2:13

P.de Felip, G.A. Luke, L.E. Hughes, D.Gani, C. Halpin and M.D.Ryan. *E unum pluribus*: multiple proteins from a self-processing polyprotein. TRENDS in Biotechnology, (2006) 24:68-75

R.F. Doll, J.E.Crandall, C.A.Dyer, J.M.Aucioin, and F.I.Smith. Comparison of promoter strengths on gene delivery into mammalian brain cells using AAV vectors. Gene Therapy, (1996) 3:437-447

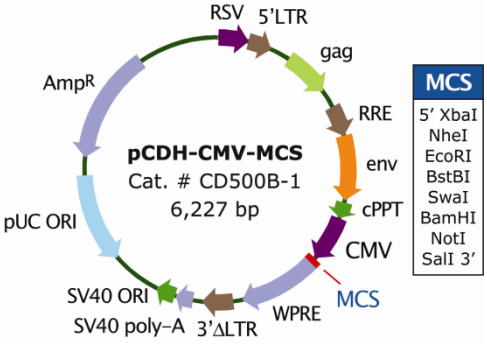
W. C. Heiser (Edit). Methods in Molecular Biology. Volume 246. Gene delivery to mammalian cells (2004), Humana Press.

X.Yu, X. Zhan, J.D'Costa, V.M. Tanavde, Z.Ye, T. Peng, M.T. Malehorn, X. Yang, C.I. Civin, and L.Cheng. lentiviral vectors with two independent internal promoters transfer high-level expression of multiple transgenes to human hematopoietic stem-progenitor cells. Molecular Therapy, (2003) 7:827-838

V. Appendix

A. Maps and Features for pCDH Vectors

1. pCDH-CMV-MCS (CD500B-1)



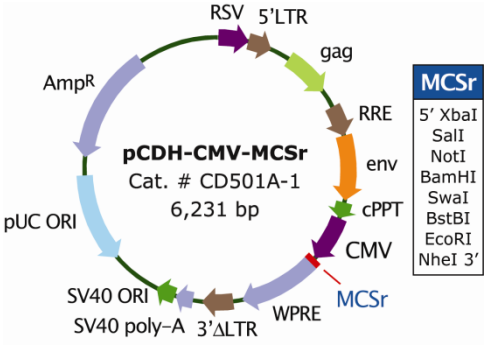
Features	
RSV:	7 - 234
5'LTR:	235 - 414
Gag:	567 - 919
RRE:	1076 - 1308
Env:	1309 - 1797
cPPT:	1798 - 1916
CMV:	1922 - 2271
WPRE:	2322 - 2912
3ΔLTR:	2984 - 3217
SV40polyA:	3289 - 3420
SV40 ORI:	3429 - 3575
pUC ORI:	3945 - 4618(c)
AmpR:	4763 - 5623(c)

5'LTR-to-3'LTR: 2,983 bp

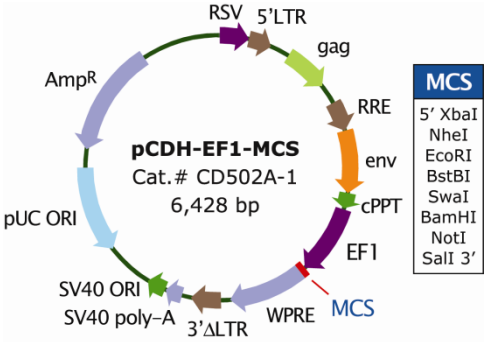
2. pCDH-CMV-MCSr (CD501A-1)

Features	
RSV:	7 - 234
5'LTR:	235 - 414
Gag:	567 - 919
RRE:	1076 - 1308
Env:	1309 - 1797
cPPT:	1798 - 1916
CMV:	1922 - 2271
WPRE:	2326 - 2916
3ΔLTR:	2988 - 3221
SV40polyA:	3293 - 3424
SV40 ORI:	3433 - 3579
pUC ORI:	3949 - 4622(c)
AmpR:	4767 - 5627(c)

5'LTR-to-3'LTR: 2,987 bp



3. pCDH-EF1-MCS (CD502A-1)

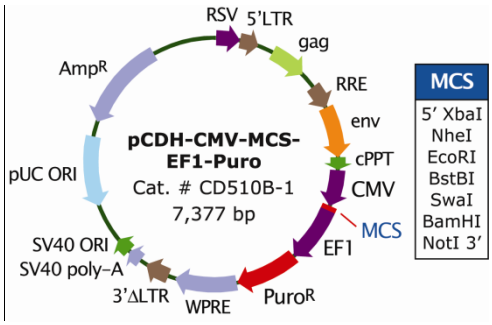


Features

RSV:	7 - 234
5'LTR:	235 - 414
Gag:	567 - 919
RRE:	1076 - 1308
Env:	1309 - 1797
cPPT:	1798 - 1916
EF1:	1928 - 2473
WPRE:	2523 - 3113
3ΔLTR:	3185 - 3418
SV40polyA:	3490 - 3621
SV40 ORI:	3630 - 3776
pUC ORI:	4146 - 4819(c)
AmpR:	4964 - 5824(c)

5'LTR-to-3'LTR: 3,184 bp

4. pCDH-CMV-MCS-EF1-Puro (CD510B-1)

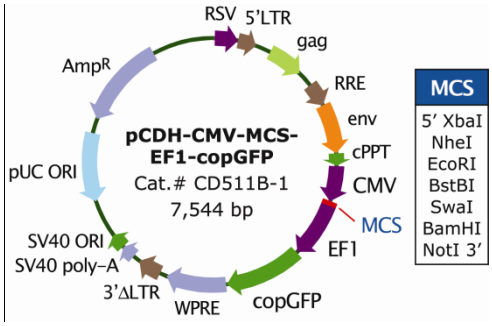


Features

RSV:	7 - 234
5'LTR:	235 - 414
Gag:	567 - 919
RRE:	1076 - 1308
Env:	1309 - 1797
cPPT:	1798 - 1916
CMV:	1922 - 2271
EF1:	2315 - 2860
PuroR:	2866 - 3462
WPRE:	3472 - 4062
3ΔLTR:	4134 - 4367
SV40polyA:	4439 - 4570
SV40 ORI:	4579 - 4725
pUC ORI:	5095 - 5768(c)
AmpR:	5913 - 6773(c)

5'LTR-to-3'LTR: 4,132 bp

5. pCDH-CMV-MCS-EF1-copGFP (CD511B-1)



Features

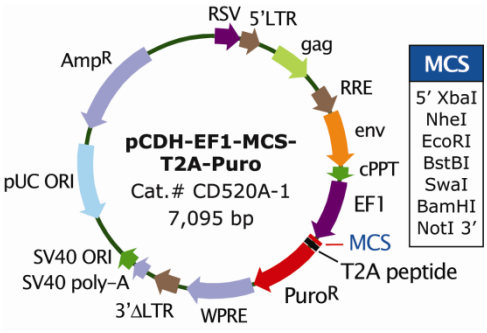
RSV:	7 - 234
5'LTR:	235 - 414
Gag:	567 - 919
RRE:	1076 - 1308
Env:	1309 - 1797
cPPT:	1798 - 1916
CMV:	1922 - 2271
EF1:	2315 - 2860
copGFP:	2874 - 3629
WPRE:	3639 - 4229
3ΔLTR:	4301 - 4534
SV40polyA:	4606 - 4737
SV40 ORI:	4746 - 4892
pUC ORI:	5262 - 5935(c)
AmpR:	6080 - 6940(c)

5'LTR-to-3'LTR: 4,300 bp

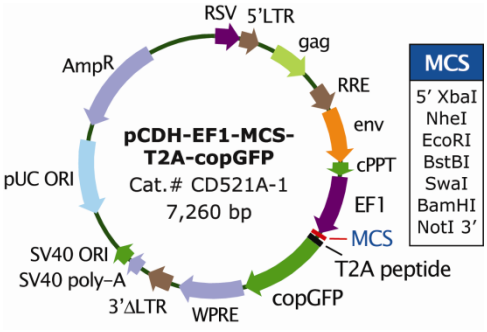
6. pCDH-EF1-MCS-T2A-Puro (CD520A-1)

Features

RSV:	7 - 234
5'LTR:	235 - 414
Gag:	567 - 919
RRE:	1076 - 1308
Env:	1309 - 1797
cPPT:	1798 - 1916
EF1:	1928 - 2473
T2A peptide:	2518 - 2571
PuroR:	2578 - 3174
WPRE:	3190 - 3780
3ΔLTR:	3852 - 4085



7. pCDH-EF1-MCS-T2A-copGFP (CD521A-1)

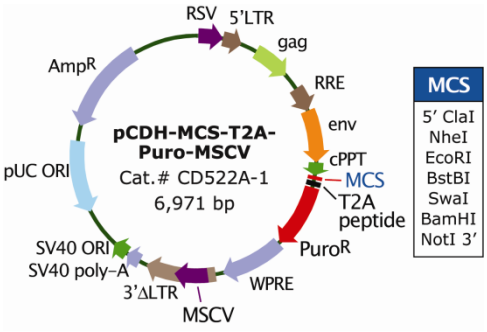


Features

RSV:	7 - 234
5'LTR:	235 - 414
Gag:	567 - 919
RRE:	1076 - 1308
Env:	1309 - 1797
cPPT:	1798 - 1916
EF1:	1928 - 2473
T2A peptide:	2518 - 2571
copGFP:	2578 - 3333
WPRE:	3355 - 3945
3ΔLTR:	4017 - 4250
SV40polyA:	4322 - 4453
SV40 ORI:	4462 - 4608
pUC ORI:	4978 - 5651(c)
AmpR:	5796 - 6656(c)

5'LTR-to-3'LTR: 4,016 bp

8. pCDH-MCS-T2A-Puro-MSCV (CD522A-1)

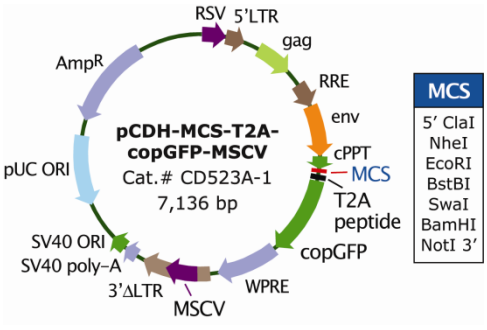


Features

RSV:	7 - 234
5'LTR:	235 - 414
Gag:	567 - 919
RRE:	1076 - 1308
Env:	1309 - 1797
cPPT:	1798 - 1916
T2A peptide:	1972 - 2025
PuroR:	2032 - 2628
WPRE:	2644 - 3234
3ΔLTR:	3306 - 3961
MSCV:	3344 - 3755
SV40 polyA:	4033 - 4164
SV40 ORI:	4173 - 4319
pUC ORI:	4689 - 5362(c)
AmpR:	5507 - 6367(c)

5'LTR-to-3'LTR: 3,961 bp

9. pCDH-MCS-T2A-copGFP-MSCV (CD523A-1)



Features

RSV:	7 - 234
5'LTR:	235 - 414
Gag:	567 - 919
RRE:	1076 - 1308
Env:	1309 - 1797
cPPT:	1798 - 1916
T2A peptide:	1972 - 2025
copGFP:	2032 - 2787
WPRE:	2809 - 3399
3ΔLTR:	3471 - 4126
MSCV:	3509 - 3920
SV40polyA:	4198 - 4329
SV40 ORI:	4338 - 4484
pUC ORI:	4854 - 5527(c)
AmpR:	5672 - 6532(c)

5'LTR-to-3'LTR: 3,892 bp

B. Descriptions of Features in pCDH Vectors

Feature	Function
3' ΔLTR (ΔU3)	Required for viral reverse transcription; self-inactivating 3' LTR with deletion in U3 region prevents formation of replication-competent viral particles after integration into genomic DNA
Amp^R	Ampicillin resistant gene for selection of the plasmid in *E. coli*
CMV promoter	Constitutive Human cytomegalovirus (CMV) promoter for transcription of reporter and/or cloned cDNA insert
copGFP	Copepod green fluorescent protein (similar to regular EGFP, but with brighter color) as a reporter for the transfected/transduced cells
cPPT	Central polypurine tract (includes DNA Flap region) involved in nuclear translocation and integration of transduced viral genome
EF1 promoter	Constitutive Elongation factor 1α promoter for transcription of reporter and/or cloned cDNA insert
env	Packaging signal
gag	Packaging signal
MSCV	Constitutive LTR enhancer/promoter of murine stem cell virus (MSCV) for transcription of reporter and/or cloned cDNA insert
pUC ORI	Allows for high-copy replication in *E. coli*
Puro^R	Puromycin-resistant marker for selection of the transfected/transduced cells
RRE	Rev response element binds gag and involved in packaging of viral transcripts
RSV / 5'LTR	Hybrid RSV promoter-R/U5 long terminal repeat; required for viral packaging and transcription
SV40 ORI	Allows for episomal replication of plasmid in eukaryotic cells
SV40 Poly-A	Transcription termination and polyadenylation
T2A Peptide	The "self-cleaving" 2A peptide mediates protein cleavage from a single open reading frame to generate multiple proteins from a single promoter
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element—enhances the stability of the viral transcripts

C. Properties of the copGFP Fluorescent Protein

The pCDH copGFP Vectors contain the full-length copGFP gene with optimized human codons for high level of expression of the fluorescent protein from the CMV, EF1, or MSCV promoter in mammalian cells. The copGFP marker is a novel natural green monomeric GFP-like protein from copepod (*Pontellina sp*.). The copGFP protein is a non-toxic, non-aggregating protein with fast protein maturation, high stability at a wide range of pH (pH 4-12), and does not require any additional cofactors or substrates. The copGFP protein has very bright fluorescence that exceeds at least 1.3 times the brightness of EGFP, the widely used *Aequorea victoria* GFP mutant. The copGFP protein emits green fluorescence with the following characteristics:

emission wavelength max – 502 nm;
excitation wavelength max – 482 nm;
quantum yield – 0.6;
extinction coefficient – 70,000 M^{-1} cm^{-1}

Due to its exceptional properties, copGFP is an excellent fluorescent marker which can be used instead of EGFP for monitoring delivery of lentivector constructs into cells.

D. Related Products

- **pPACKH1™ Lentivector Packaging Kit (Cat. # LV500A-1)**
  Unique lentiviral vectors that produce all the necessary HIV viral proteins and the VSV-G envelope glycoprotein from vesicular stomatitis virus required to make active pseudoviral particles. 293TN cells (SBI, Cat. # LV900A-1) transiently transfected with the pPACKH1 and a pCDH cDNA expression construct produce packaged viral particles containing a pCDH cDNA construct.

- **FIV-Based pCDF cDNA Cloning and Expression Vectors**
  - **pCDF1-MCS1** (Cat. # CD100A-1)
  - **pCDF1-MCS2-EF1-Puro** (Cat. # CD110B-1)
  - **pCDF1-MCS2-EF1-copGFP** (Cat. # CD111B-1)

- **RNAi Cloning and Expression Lentivectors**
  These FIV and HIV-based single- and double-promoter shRNA and siRNA cloning vectors allow you to clone siRNA templates and efficiently transduce these siRNA constructs in a wide range of cells. For a list of currently available vectors, please visit our website at http://www.systembio.com.

- **MicroRNA Precursor Construct Collection**
  FIV-based microRNA Precursor Constructs allow you to express pre-miRNA, consisting of the stem loop structure and upstream and downstream flanking genomic sequence. For a list of currently available vectors, please visit our website at http://www.systembio.com.

- **PathNet™ Transcriptional Reporter Lentivectors**
  FIV and HIV-based transcriptional reporter vectors, allow detection of the activation of transcriptional factors (TFs) in a natural environment (nuclei). For a list of currently available vectors, please visit our website at http://www.systembio.com.

E. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

System Biosciences (SBI)
265 North Whisman Rd..
Mountain View, CA 94043

Phone: (650) 968-2200
(888) 266-5066 (Toll Free)

Fax: (650) 968-2277

E-mail:
General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

VI. Licensing and Warranty Statement

Limited Use License

Use of the pCDH cDNA Cloning and Expression Vector (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

The purchaser of the Product is granted a limited license to use the Product under the following terms and conditions:

The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use.

The Product may not be resold, modified for resale, or used to manufacture commercial products without prior written consent of SBI.

This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research.

HIV Vector System

This Product or the use of this Product is covered by U.S. Patents Nos. 5,665,577 and 5,981,276 (and foreign equivalents) owned by the Dana-Farber Cancer Institute, Inc., and licensed by SBI. This product is for non-clinical research use only. Use of this Product to produce products for resale or for any diagnostic, therapeutic, clinical, veterinary, or food purpose is prohibited. In order to obtain a license to use this Product for these commercial purposes, contact the Office of Research and Technology Ventures at the Dana-Farber Cancer Institute, Inc. in Boston, Massachusetts, USA.

WPRE Technology

System Biosciences (SBI) has a license to sell the Product containing WPRE, under the terms described below. Any use of the WPRE outside of SBI's Product or the Products' intended use, requires a license as detailed below. Before using the Product containing WPRE, please read the following license agreement. If you do not agree to be bound by its terms, contact SBI within 10 days for authorization to return the unused Product containing WPRE and to receive a full credit.

The WPRE technology is covered by patents issued to The Salk Institute for Biological Studies.

SBI grants you a non-exclusive license to use the enclosed Product containing WPRE in its entirety for its intended use. The Product containing WPRE is being transferred to you in furtherance of, and reliance on, such license. Any use of WPRE outside of SBI's Product or the Product's intended use, requires a license from the Salk Institute for Biological Studies.

This license agreement is effective until terminated. You may terminate it at any time by destroying all Products containing WPRE in your control. It will also terminate automatically if you fail to comply with the terms and conditions of the license agreement. You shall, upon termination of the license agreement, destroy all Products containing WPRE in you control, and so notify SBI in writing.

This License shall be governed in its interpretation and enforcement by the laws of California.

Contact for WPRE Licensing: The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037; Attn: Office for Technology Management; Phone: (858) 435-4100 extension 1275; Fax: (858) 450-0509.

CMV Promoter

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242.

CopGFP Reporter

This product contains a proprietary nucleic acid coding for a proprietary fluorescent protein(s) intended to be used for research purposes only. Any use of the proprietary nucleic acids other than for research use is strictly prohibited. USE IN ANY OTHER APPLICATION REQUIRES A LICENSE FROM EVROGEN. To obtain such a license, please contact Evrogen at license@evrogen.com.

SBI has pending patent applications on various features and components of the Product. For information concerning licenses for commercial use, contact SBI.

Purchase of the product does not grant any rights or license for use other than those explicitly listed in this Licensing and Warranty Statement. Use of the Product for any use other than described expressly herein may be covered by patents or subject to rights other than those mentioned. SBI disclaims any and all responsibility for injury or damage which may be caused by the failure of the buyer or any other person to use the Product in accordance with the terms and conditions outlined herein.

Limited Warranty

SBI warrants that the Product meets the specifications described in the accompanying Product Analysis Certificate. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

SBI's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

SBI is committed to providing our customers with high-quality products. If you should have any questions or concerns about any SBI products, please contact us at (888) 266-5066.

© 2012 System Biosciences (SBI).